

Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology

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Adoptive T cell transfer for cancer and chronic infection is an emerging field that shows promise in recent trials. Synthetic-biology-based engineering of T lymphocytes to express high-affinity antigen receptors can overcome immune tolerance, which has been a major limitation of immunotherapy-based strategies. Advances in cell engineering and culture approaches to enable efficient gene transfer and ex vivo cell expansion have facilitated broader evaluation of this technology, moving adoptive transfer from a “boutique” application to the cusp of a mainstream technology. The major challenge currently facing the field is to increase the specificity of engineered T cells for tumors, because targeting shared antigens has the potential to lead to on-target off-tumor toxicities, as observed in recent trials. As the field of adoptive transfer technology matures, the major engineering challenge is the development of automated cell culture systems, so that the approach can extend beyond specialized academic centers and become widely available.

Introduction

Adoptive T cell transfer involves the isolation and reinfusion of T lymphocytes into patients to treat disease. The ultimate objective of the process is conceptually the same as that of a successful T cell immunization, namely the stimulation and expansion of potent and antigen-specific T cell immunity. Adoptive T cell transfer additionally offers the potential to overcome one of the significant limitations associated with vaccine-based strategies, specifically the requirement to de novo activate and expand a tumor antigen-specific T cell response in patients who are often immune compromised and deeply tolerant to cancer antigens or to antigens that are expressed during chronic infection.

Targeting of disease through the adoptive transfer of lymphocytes was first reported more than 50 years ago in rodent models (Mitchison, 1955). Improved understanding of T cell biology, including the mechanisms for T cells activation and recognition of targets, the role of accessory surface molecules and signal transduction pathways involved in the regulation of T cell function and survival, as well as the identification and cloning of soluble T cell growth factors, has facilitated the ability to expand ex vivo large numbers of T cells for adoptive immunotherapy. There are several excellent reviews of the rationale and experimental basis for adoptive T cell therapy of tumors (Cheever and Chen, 1997; Greenberg, 1991; Restifo et al., 2012).

Significant effort has been extended over the past few years to evaluate the potential for adoptive T cell transfer to treat cancer. A number of strategies have been evaluated, initially using T cells isolated from tumor-infiltrating lymphocytes (TILs) (Dudley et al., 2008). Adoptive transfer of bulk T lymphocytes, obtained from the periphery and expanded ex vivo to generate large numbers prior to reinfusion into patients, is an alternative strategy for adoptive T cell therapy (Rapoport et al., 2005). Initial approaches to apply this strategy involved leukapheresis of peripheral blood mononuclear cells (PBMCs) from patients followed by bulk ex vivo expansion and reinfusion along with exogenous interleukin-2 (IL-2). This approach does not specifically enrich for an-

tigen-specific T cells, but rather generates a population of activated T cells with lowered triggering thresholds. Clinical trials to evaluate the potential of adoptively transferred autologous activated T cells to augment stem cell transplants for hematologic malignancies showed that infusion of autologous costimulated T cells resulted in a rapid reconstitution of lymphocyte numbers (Laport et al., 2003) and randomized trials demonstrated that expanded cells were functional (Rapoport et al., 2005). Data from more recent clinical trials using engineered antigen-specific T cells have started to reveal the full potential of adoptive T cell therapy to effectively target cancer, with objective clinical activity in a number of cases (Brentjens et al., 2013; Johnson et al., 2009; Kochenderfer et al., 2012) including complete and long-lasting durable clinical responses observed in patients with late-stage, chemotherapy-resistant leukemias (Grupp et al., 2013; Kalos et al., 2011). These recent results have shown that it is possible to achieve a long-standing objective of adoptive T cell therapy. This objective has been to recapitulate the end result of a successful T cell vaccine, with robust T cell expansion in vivo, potent antitumor activity, T cell contraction, and long-term functional persistence of a memory T cell subset. However, we propose that the goal with engineered T cells is not simply to recapitulate T cell vaccines, but rather to use the emerging discipline of synthetic biology, which combines elements of engineering and molecular biology to create new immune systems with enhanced functionalities (Chen et al., 2012). In this regard, the principles of gene transfer combined with adoptive cellular therapy are poised to overcome the fundamental limitations associated with central and peripheral tolerance and enable the potent and efficient “at-will” targeting of tumors. In this article we summarize the state of the art and highlight outstanding issues for the effective application of engineered T cell therapy to treat cancer.

Using Bispecific T Cells to Overcome Tolerance

The great majority of to-date targeted tumor antigens are self-antigens, normally expressed during development and

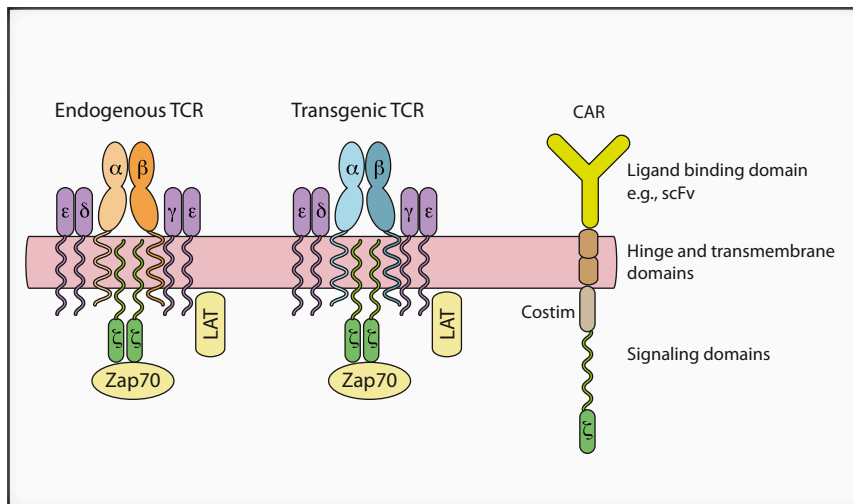


Figure 1. Engineered T Cells that Have Retargeted Specificity

Bispecific and trispecific T cells are created by introduction of genes that encode TCRs and CARs of desired specificity and affinities for tumors. The T cells retain expression of the endogenous TCR, unless this is knocked down by various approaches. CARs target surface antigens in an MHC-independent fashion. Abbreviations are as follows: Costim, cosignaling domain such as CD28 or 4-1BB; LAT, linker for activation of T cells; scFv, single-chain variable fragment; ZAP70, zeta chain associated protein kinase 70 kDa.

Rational high-throughput genetic mutagenesis approaches have been applied to enhance the affinity of tumor-antigen-specific $\alpha\beta$ TCR (Chervin et al., 2008; Li et al., 2005), and such efforts have resulted in the ability to molecularly engineer $\alpha\beta$ TCR with substantially higher

aberrantly expressed by tumors. The striking difference in affinity between T cell receptors specific for self-antigens expressed by tumors and T cell receptors specific for virus antigens has been summarized recently; these comparative analyses have revealed that TCR from T cells that recognize self-tumor antigens have substantially lower affinities (approximately 1.5 logs) for cognate MHC:peptide complexes compared to their virus-specific counterparts (Aleksic et al., 2012; Cole et al., 2007). This observation, which reflects the impact of central tolerance on the T cell repertoire to self-antigens, points to the fact that T cells that escape central tolerance and have the potential to respond to the self-target antigens will manifest in most cases suboptimal activation in terms of antitumor activity. The high tolerance to tumor antigens with normal and/or developmental expression combined with the potent immunosuppressive microenvironment often present at the tumor site support the premise that enhancing antitumor immunity by the adoptive transfer of “native” T cells may not be sufficient to induce tumor cell death in most patients with advanced malignancy.

Gene-transfer-based strategies have been developed to overcome the consequences of immune tolerance on the tumor-specific T cell repertoire. These approaches provide the potential to redirect T cells to effectively target tumors by the transfer of antigen-specific T cell receptor α and β chains ($\alpha\beta$ TCR) or chimeric antigen receptors (CARs) composed of antibody binding domains fused to T cell signaling domains. In each case recipient T cells acquire the engineered, tumor-specific specificity while retaining their initial specificity (Figure 1).

The primary source for isolating tumor-specific $\alpha\beta$ TCR has been tumor-specific T cell clones obtained from cancer patients or healthy volunteers. A number of approaches have been developed to isolate such T cells based on in vitro stimulation strategies by peptides or whole antigen. These approaches have in general been tedious and inefficient, at least in part because of the lack of robust in vitro expansion protocols and the low frequency of such T cells in peripheral blood. Additionally, because of the impact of central tolerance on the T cell repertoire, T cells isolated by these approaches have typically been of low affinity with at best modest antitumor activity.

affinities for target antigens (Li et al., 2005). Alternative strategies to improve $\alpha\beta$ TCR avidity by engineering TCR chains have also been pursued (Kuball et al., 2009).

Affinity-enhanced $\alpha\beta$ TCR-based engineering approaches have certain inherent biological advantages, most notably that essentially all cellular proteins can be targeted because the approach is not limited to the targeting of cell surface epitopes and the primary T cell activation signal is delivered in a physiological context, which may be relevant for optimal functionality of the infused T cells. On the other hand, this approach suffers from certain disadvantages; in particular, $\alpha\beta$ TCR-based targeting approaches remain susceptible to the common tumor escape mechanisms of MHC downmodulation and altered peptide processing; furthermore, the mutagenesis process has the potential to result in the generation of neo-epitopes that can become the target of humoral and cellular immune responses in patients.

An additional concern for the broader implementation of affinity-enhanced $\alpha\beta$ TCR relates to the development of secondary and potentially deleterious specificities as a consequence of the mutagenesis and enhanced affinity process (Zhao et al., 2007). As discussed in more detail below, establishing robust and systematic strategies to evaluate this potential on a case-by-case basis will be critical for broader implementation of this approach.

Engineering of T cells by the introduction of chimeric antigen receptors (CARs) is an alternative approach to redirect T cell specificity (Gross et al., 1989). CARs are synthetic polypeptides that contain three distinct modules: an extracellular target binding module, a transmembrane module that anchors the molecule into the cell membrane, and an intracellular signaling module that transmits activation signals. The target binding module is usually generated by scFv determinants isolated from antibodies, linked in a single chain through linker polypeptide sequences. Transmembrane modules are most commonly derived from molecules involved in T cell function such as CD8 and CD28. The intracellular module almost always consists of the zeta chain of the TCR complex responsible for transmitting TCR engagement-mediated activation signals to cells. As discussed in more detail

below, more recently developed CARs incorporate additional domains associated with T cell functions in efforts to augment zeta signaling in a physiologically relevant manner.

CAR-based strategies provide distinct advantages in terms of redirecting effective antitumor activity: because the target-binding moiety is derived from antibodies with affinities several orders of magnitude higher than $\alpha\beta$ TCR, CAR-engineered cells bypass the fundamental issue of central tolerance. Because CARs recognize intact cell surface proteins, targeting of target cells is neither MHC restricted nor dependent on processing and effective presentation of target epitopes, and consequently CAR-based approaches are insensitive to tumor escape mechanisms related to HLA downmodulation and altered processing escape mechanisms, an issue that is distressingly common in human carcinoma (Vitale et al., 2005). On the other hand, a limitation of the CAR-based approach is that they are restricted to targeting of cell surface determinants. A recent report has described the development of CARs that bind to the intracellular antigen WT-1 via antibody scFv domains that bind to MHC class I:peptide complexes (Dao et al., 2013). Finally, because CARs are chimeric molecules that include unique junctional fragments and murine sequences in the scFv domains, CAR-modified T cells can be targeted by patient humoral and cellular immune responses.

Approaches to Genetically Engineer Lymphocytes

Advances in basic molecular biology have precipitated a number of approaches to engineer lymphocytes at the genomic, RNA, epigenetic, and protein levels, with the goal of pharmacologically enhancing the immune system. Approaches to engineer lymphocytes have been reviewed previously (June et al., 2009). The combination of diverse approaches to effectively engineer T cells combined with recently acquired mechanistic insights into T cell biology and tumor immunity have converged to the point where the rational engineering of potent antitumor T cell immunity is a practical and clinically testable reality. The majority of clinical approaches have employed T cells engineered to stably express transgenes via virus-based transduction. Virus-mediated gene transfer approaches typically employ vectors that are derived from gamma retroviruses or more recently lentiviruses, principally because of their ability to integrate into the host genome and drive long-term transgene expression, as well as for their low intrinsic immunogenicity. Gammaretrovirus-based transduction requires replicating cells for viral integration into genomic DNA, whereas lentiviral vectors can also integrate into nondividing cells; lentiviral vectors also appear to be less susceptible to silencing by host restriction factors and can deliver larger DNA sequences than can retroviruses (Naldini et al., 1996). Although virus-based approaches result in reasonably efficient transduction of primary T cells, they have considerable limitations in terms of cost to manufacture clinical-grade material, the total size of DNA that can be included in the virus vectors, and the potential, principally for retroviruses, for the integration events to result in insertional oncogenesis. A new virus-based system that has not yet entered clinical trials is based on foamy virus vectors, which possess favorable integration properties and are not pathogenic in humans (Williams, 2008). Non-virus-based approaches benefit from lower manufacturing cost and are in principle less immunogenic than viral ap-

proaches. Although such approaches are theoretically safer because they are not dependent on viral elements integrating into host DNA, their safety record is shorter than that of virus-based vectors. Nonviral approaches to introduce transgenes into T cells involve the utilization of transposon elements such as sleeping beauty and piggybac as well as zinc-finger nuclease, TALEN, and CRISPR/Cas9 based-technologies, which allow for the ability to engineer T cell populations with transgene insertions into specific chromosomal loci or that are biallelically disrupted for specific genes (Hackett et al., 2010; Perez et al., 2008; Reyon et al., 2012). Such technologies offer significant potential to be able to engineer T cells in a manner that allows for the ability to interrupt or otherwise modulate expression of particular proteins that may be deleterious to therapeutic function.

As discussed above, a potential safety concern related to the infusion of engineered T cells is integration-related insertional mutagenesis and cellular transformation, which has been demonstrated with the genetic engineering of hematopoietic stem cells (Hacein-Bey-Abina et al., 2008). Retrovirally modified T cells have been shown to persist for more than a decade without adverse effects after adoptive transfer in patients with congenital and acquired immunodeficiency (Muul et al., 2003; Scholler et al., 2012), indicating that the retrovirus-based approaches to genetically modify mature human T cells are fundamentally safe. There are less data available on use of lentiviruses, but to date no safety concerns have been raised, and analysis of transduced T cells recovered from patients indicates that the lentiviral integration sites are not random and do not favor proto-oncogenes or tumor suppressor genes (Bushman, 2007; Wang et al., 2009).

For some applications, permanent genomic alteration may not be necessary for therapeutic efficacy, or transient expression may be required to mitigate potential unanticipated toxicity. In situations where transient gene transfer is required, nonintegrating viruses, such as the adenovirus-based Ad5-35 vectors, can achieve high efficiencies of gene transfer to human T cells (Perez et al., 2008). One emerging alternative to virus-mediated gene transfer approaches is RNA transfection. Primary human T lymphocytes transiently express proteins for at least a week after electroporation via in vitro-transcribed mRNA, and such engineered cells mediate potent and antigen-specific effector functions (Zhao et al., 2010). RNA electroporation has been used to deliver message for TCRs or CARs, chemokine receptors or cytokines (Mitchell et al., 2008; Rowley et al., 2009; Yoon et al., 2009). Because RNA electroporation is a cost-effective and efficient mechanism to engineer T cells, this approach is attractive for high-throughput and iterative testing of novel constructs and/or targets, and clinical trials using mRNA-electroporated lymphocytes are ongoing at several centers. The transient nature of RNA-based strategies provides considerable safety advantages, particularly when exploring the potential to target antigens not previously evaluated by CAR technology and where low-level expression in normal tissues may be problematic in terms of toxicity. Finally, the flexibility of this platform allows for the ability to cotransfer multiple transcripts and augment T cell function by introducing molecules that enhance costimulation and effector functions, mediate homing to target tissues, or cotarget multiple antigens.

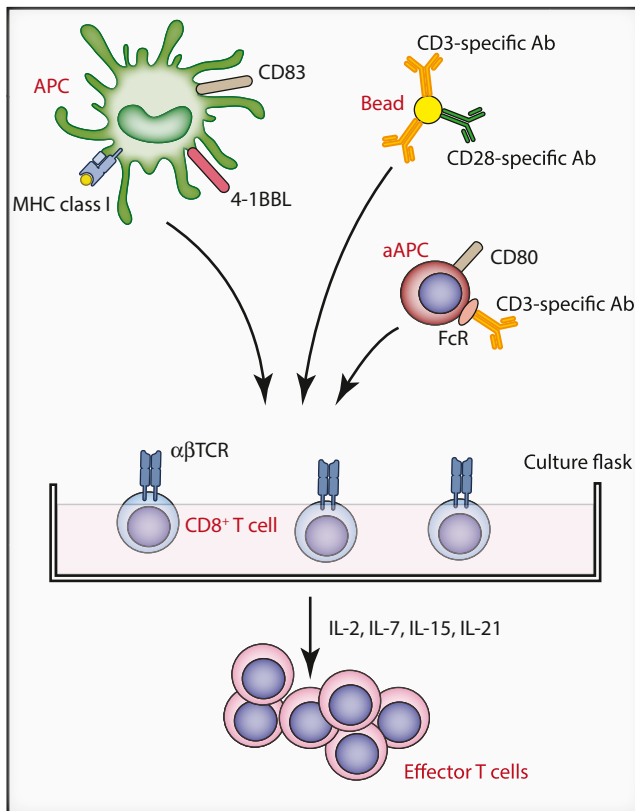


Figure 2. Cell Culture Approaches

CTLs express $\alpha\beta$ TCRs and are stimulated by APCs that express MHC class I. Feeder cells or artificial APC (aAPC) that express the costimulatory ligands 4-1BBL and CD83 or beads coated with agonistic antibodies enhance growth and function. T cells can be stimulated by beads or cell-based aAPC in the presence of various cytokines.

An additional challenge for the field is the ability to more precisely control the activity of transferred engineered cells. Initial efforts focused on developing approaches to ablate engineered cells through the introduction of “suicide genes” such as herpes simplex virus thymidine kinase (TK); these initial efforts demonstrated the potential for immune-based rejection as a result of targeting TK-derived sequences (Marktel et al., 2003). More recently, an inducible system based on a fusion protein comprised of an extracellular FK506 binding domain linked to human caspase-9 signaling domains to deliver apoptotic signals in response a small molecule-mediated dimerization has been developed and is currently being evaluated in clinical trials (Di Stasi et al., 2011). Another potential approach is to engineer T cells to express signaling pathways that causes the T cell to destroy itself after a defined number of cell divisions (Friedland et al., 2009). Looking forward, the development of approaches that enable modulation of T cell function and activity rather than T cell ablation are likely to result in more precisely tuned T cell specificity and function.

Although the ability to redirect T cell specificity to target antigens of choice has perhaps been the most obvious development in the field of T cell-based gene therapy, there are other aspects of T cell biology that are amenable to genetic manipulation and probably are important to address for the successful broad

application of adoptive immunotherapy. These aspects include long-term functional persistence, trafficking of effector cells and accumulation at the tumor site, and engagement of costimulatory receptors to mediate a robust effector response. In addition, infused T cells must be able to resist mechanisms of exhaustion, senescence, and immunosuppression by the tumor microenvironment, to avoid the host humoral and cellular immune responses, and additionally to be amenable to deletion on demand to mitigate potential toxicity issues. Some examples include strategies to encode molecules involved in costimulation (Krause et al., 1998), the prevention of apoptosis (Charo et al., 2005), the remodeling of the tumor microenvironment (Kerker et al., 2011), and the induction of homeostatic proliferation (Cheng et al., 2002), as well as CARs encoding chemokine receptors that promote T cell homing (Moon et al., 2011).

Beyond Boutique: Producing Weapons of Mass Destruction for the Masses

The potential to target cancer by adoptive transfer of various lymphocyte subsets is currently being tested in numerous clinical trials. Given that the optimal cell culture conditions are not the same for distinct lymphocyte subpopulations, there are multiple approaches that are being tested for ex vivo expansion (Figure 2). For example, investigators are testing infusion of engineered CD4⁺ cells, CD8⁺ cells, Treg cells, invariant NKT cells, and gamma delta T cells. The use of engineered memory stem cells (that is, T cells programmed for the most extensive self-renewal) has significant but as-yet-not-explored potential (Gattinoni et al., 2011).

Autologous engineered cell therapies are a paradigm shift from conventional biologics such as pills, vaccines, small molecule inhibitor molecules, and antibodies in that the approach requires a patient-specific product. Some have dismissed adoptive T cell immunotherapy as a fringe or boutique therapy that would be impossible to commercialize (Baker, 2011). Indeed, several challenges must be overcome before this disruptive therapy can become broadly applicable and widely available. The barriers that we currently perceive fall into two areas. First, the cell culture systems must be robust and reproducible. The T cell engineering process that we and others have developed requires complex logistics. Some of the variables that need to be standardized in order to scale this out for widespread use include developing a leukapheresis network, standardizing and scaling up the manufacturing of lentiviral vectors, and developing validated cell-shipping and chain-of-custody procedures. For example, the cell culture media that will be used for commercial scale must be serum free because there is an insufficient supply of bovine or human-derived serum to support large-scale manufacturing (Brindley et al., 2012). Second, personalized cell therapies cannot become widely available if the cell culture process requires extensive manipulation by highly skilled scientists and technicians (Mason and Manzotti, 2010). Therefore, automated culture systems need to be developed. There is precedent in the automotive industry, where cars were initially manufactured in assembly lines, but manually. Today’s automobiles are assembled largely by robots and other forms of automation (Michalos et al., 2010). As engineered T cell processing becomes more automated, cell products will be produced for greater number of patients more efficiently. Given the recent entry of the

pharmaceutical industry to this field, we are optimistic that the resources and expertise of the pharmaceutical industry will create the infrastructure required for the widespread availability of this disruptive technology. Further clinical development of engineered T cell therapies in large numbers of patients will be challenging but is justified given the magnitude of therapeutic effects recently observed.

Lessons from Clinical Trials

Poor *in vivo* persistence has been a repeating theme in most T cell adoptive transfer trials where lack of overall efficacy was observed. Low persistence of infused T cells may be influenced by a number of variables, including *ex vivo* culture conditions, lack of long-term transgene expression, poor effector functionality, exhaustion and replicative senescence, and the development of anti-infused cell humoral and/or cellular immune responses. Data generated from both preclinical models and clinical trials have highlighted a few critical considerations likely to be important for the ultimately effective clinical development of T cell therapy.

Animal studies have clearly shown that some T cell subtypes show desirable qualities *in vivo* such as enhanced engraftment, antitumor effect, or survival. A controversy exists as to whether one subset is preferable; there are reports favoring naive T cells (Hinrichs et al., 2009), central memory T cells (Berger et al., 2008), Th17 cells (Muranski et al., 2008), and so-called memory stem cells (Gattinoni et al., 2011). Despite these preclinical observations, there are as yet no direct clinical comparisons of the infusions of T cell subsets alone or as a bulk population. A related controversy is that although it is tempting to try to select a particular subtype (e.g., CD8⁺ cytotoxic lymphocytes) for adoptive cellular therapy, there is a risk inherent to applying a reductionist approach to the immune system. For example, there is compelling evidence that CD4⁺ T cells are required for CD8⁺ memory formation, clearly an important quality in the control of tumor (Sun and Bevan, 2003; Sun et al., 2004). Cell selection schemata and culture conditions can be optimized to promote the expansion of T central memory cells (Wang et al., 2012).

It is now well recognized that stimulation of T cells via their TCR without a second costimulatory signal induces tolerance and therefore more recent CAR-based technologies have focused on overcoming this limitation. Thus, although first-generation CARs depended on intracellular transduction of the recognition signal via the CD3 ζ chain alone, second- and third-generation CAR constructs have incorporated second, typically costimulatory, signaling domains such as those derived from CD28, ICOS, CD134, or CD137 (Sadelain et al., 2013).

Recent reports from clinical trials that used CAR T cells with CD137 and TCR zeta signaling domains, which documented long-term functional persistence of T cells engineered to target CD19, along with long-lasting clinical remissions and ongoing B cell aplasia, have highlighted the potential for adoptive T cell transfer to effect profound long-term functional antitumor activity (Grupp et al., 2013; Kalos et al., 2011). Most previous clinical trials utilizing engineered T cells were characterized by poor persistence of the infused product, and the generally disappointing clinical results were probably attributed to inherently poor proliferative capacity or to rejection by the host of the transferred product (Kershaw et al., 2006; Park et al., 2007).

Expression of costimulatory molecules *in trans* with CARs has also been recently demonstrated (Topp et al., 2003), opening up the possibility for new modular engineering designs. Ongoing efforts by a number of groups have focused on the evaluation of signaling domains that have the potential to generate qualitatively unique T cell populations that have the potential to drive T cells to differentiate into unique subsets, such as signaling domains from ICOS that drive human T cells to a Th17 cell phenotype (Paulos et al., 2010). Additional strategies have involved the overexpression in T cells of prosurvival signals such as telomerase (Rufer et al., 2001), antiapoptotic genes (Eaton et al., 2002), and the downregulation of proapoptotic molecules such as Fas (Dotti et al., 2005). Yet another approach to enhance T cell survival involves the expression of dominant-negative receptors for inhibitory molecules such as dominant-negative receptors for TGF- β (Bollard et al., 2002). Recent data indicate that effector-like CD27-negative CD8 cells mediate potent protective immunity and lead to long-lived memory (Olson et al., 2013). It is likely that CAR T cells “rewire” their signaling and that the cells that persist as memory cells are different from those in a natural immune response. Because nonphysiologic approaches that enhance T cell proliferation and survival have the potential to generate T cells that do not depend on antigen-specific engagement for survival, each of these approaches needs to be developed in concert with parallel approaches to track and mitigate safety concerns.

Exogenous cytokine administration has been reported to enhance persistence of adoptively transferred CTLs (Yee et al., 2002). The cytokine most studied in this regard is IL-2, which has long been utilized as a T cell growth factor and considered to be essential in adoptive therapy protocols that involved transfer of CD8⁺ T cells. Recent studies show that although IL-2 induces proliferation of effector CD8⁺ T cells, it may be deleterious to the persistence of memory T cells. IL-2 has also been reported to increase the number of Treg cells (Zhang et al., 2005), which may be a consequence of the high levels of expression of CD25, the IL-2 receptor α chain by these cells (Ma et al., 2006). Studies that have tested the coadministration of CD4⁺ T and CD8⁺ CAR T cells have demonstrated that T cell persistence is not increased by coadministration of exogenous IL-2, presumably reflecting the helper functions of the CD4⁺ cell subset (Mitsuyasu et al., 2000). Other common gamma-chain cytokines such as IL-7 and IL-15 are also important for T cell expansion and activation and have shown potential as important modulators of T cell proliferation, differentiation, and function *in vivo*. IL-15 and IL-7 may select for the persistence of memory CD8⁺ T cells and decrease the relative number of Treg cells in mice (Ku et al., 2000) and nonhuman primates (Berger et al., 2009). IL-15 appears to induce constitutive telomerase activity in T cells, which may result in enhanced survival of engineered T cells by virtue of delaying telomere loss (Hsu et al., 2007). Advances in T cell engineering allow for the codelivery of cytokines or cytokine receptors into T cells, facilitating autocrine proliferative responses (Evans et al., 1999).

A potential limitation, particularly with the targeting of solid tumors, is the effective trafficking of engineered T cells to sites of disease. Considerable work to unravel mechanisms for T cell trafficking has revealed soluble factors, receptors, and adhesion molecules important for mediating T cell trafficking (Nolz et al.,

2011), and early work to engineer T cells to express chemokine receptors has highlighted the considerable promise of this approach to enhance antitumor activity (Mitchell et al., 2008; Moon et al., 2011). Critical and significant challenges remain with regard to understanding the mechanisms and obstacles that impact effective T cell infiltration of solid tumors (Fisher et al., 2006; Harlin et al., 2009). Fever enhances the adherence of T cells to the tumor microvasculature (Fisher et al., 2011), so that the induction of hyperthermia may augment efficacy of adoptive T cell transfer; parenthetically, some of the beneficial effects reported after IL-2 coadministration with T cells may be due to pyrogenic effects.

A significant obstacle to overcome is the exhaustion of T cells in the immune-suppressive milieu within the tumor microenvironment (Baitsch et al., 2011). A promising avenue of future research will involve the evaluation of combination therapies that combine the adoptive transfer of T cells with the increasing arsenal of immunomodulatory agents that target T cell inhibitory molecules such as CTLA-4 and PD-1 (Quezada et al., 2010).

Ex vivo manipulation of T cell products to enhance potency has the potential to make the product more immunogenic after transfer because such manipulations commonly introduce engineered, i.e., nonphysiologic, sequences into the recipient cells. Because most CARs are derived from murine anti-human scFv, there is the potential for development of both humoral and cellular responses against CAR sequences or epitopes from the retroviral vector backbone, and these responses correlate with rejection and disappearance of the infused cells from the circulation (Davis et al., 2010; Lamers et al., 2011). Recently, anaphylaxis was reported in a patient after infusion of CAR-engineered cells that targeted mesothelin through murine-derived scFv (Maus et al., 2013). Anaphylaxis has also been reported after the infusion of autologous human cells cultured in bovine serum albumin (Macy et al., 1989). Although use of serum-free cell culture techniques has reduced the potential immunogenicity associated with the use of xenobiotic sera, there is still the potential for non-self translated open reading frames present in vector sequences leading to rejection of infused cells. This phenomenon has been demonstrated in a number of cases (Berger et al., 2006; Jensen et al., 2010).

Host Conditioning and Lymphodepletion

Immunodepletion with chemoradiotherapy is critical to enhance engraftment and efficacy of adoptively transferred T cells in lymphoma and melanoma (Dudley et al., 2002; Laport et al., 2003). These results are probably due to multiple mechanisms, based on studies in mice (Klebanoff et al., 2005).

Data from both preclinical studies and clinical trials have led to the conclusion that host lymphodepletion prior to T cell infusion is important to enhance the efficacy of adoptively transferred T cells (Brentjens et al., 2011; Pegram et al., 2012). Lymphodepletion is thought to facilitate T cell expansion and persistence through the creation of homeostatic space for T cell expansion and the depletion of cytokine sinks in the form of other immune cells, leading to the induction of a memory phenotype and enhanced effector functionality (Dudley et al., 2002; Muranski et al., 2006; Wrzesinski and Restifo, 2005). Host conditioning in the form of chemo- or radiotherapy is thought to facilitate T cell engraftment and survival through the production of homeo-

static cytokines such as IL-15 as a consequence of the inflammatory reaction (Miller et al., 2005). The timing of lymphodepletion and conditioning relative to T cell infusion, and whether particular conditioning regimens are superior is also unclear; particular regimens have been shown to preferentially induce immunogenic cell death (Machiels et al., 2001; Obeid et al., 2007). With regard to timing of the infusion, recent data appear to suggest that T cell infusion early after conditioning is optimal. In both adult patients and in a pediatric population we find that infusion on day +2 is superior to infusions at later time points in terms of functional immune reconstitution after high-dose chemotherapy and stem cell infusion (Grupp et al., 2012; Rapoport et al., 2009).

In terms of the optimal schedule and dose of T cell infusion, information in the field is divergent. Whether it is preferable for T cells to be infused in a single dose or in a fractionated schedule is unclear. Data from animal models suggest that in the absence of lymphodepletion, multiple infusions are superior to a single infusion of T cells (Kircher et al., 2003). Data from bone marrow transplantation studies suggest that a fractionated infusion schedule is safer (Peggs et al., 2004); fractionation also affords the potential to mitigate the severity of toxicity in case of infusion-related adverse events. Optimal dosing of T cells, typically reported as total number of viable cells infused based on actual body weight or surface area, is complicated by age-related variability in total lymphocyte numbers and critical but to-date-unresolved variability in the replicative potential and functionality of the infused cells. Notably, the level of T cell engraftment and persistence may not correlate with the infused dose, and the temporal kinetics of maximal engraftment are not yet defined. In our studies of adoptively transferred autologous CAR T cells, we often find that the number of cells in the host peaks 2 to 3 weeks after infusion of the cells (Kalos et al., 2011). The identification of populations of T cells with "stem cell-like" properties and the recent demonstration of a plasticity of T cell subsets point to the future possibility to engineer small numbers of T cells with the potential for durable antitumor immunity (Gattinoni et al., 2011; Stemmerger et al., 2007).

Consequences of Engineering around Tolerance: Off-Target Recognition

An important evolutionary driver for the developing immune system is the establishment of a T cell repertoire with the potential to be appropriately activated by non-self in an MHC-restricted manner but to not respond inappropriately to self. Accordingly, the immune system has evolved to sculpt T cell specificity and potency with exquisite caution, with both central and peripheral tolerance mechanisms driving the process to prevent targeting of self-tissues under physiologic conditions. Engineering and manipulation of T lymphocytes to have redirected and increased potency has the potential to alter this natural balance and create T cells with the enhanced potential to be activated by self, an issue of considerable concern to the field.

The enhanced potential for self-triggering of engineered T cells can be a consequence of a number of events, such as a lowered T cell activation threshold as a result of ex vivo activation, increased sensitivity of affinity-enhanced T cells to be triggered by low levels of antigen, or through bypassing peripheral immunosuppressive mechanisms by the introduction of costimulatory

signaling domains or depletion of regulatory T cells during ex vivo expansion. Perhaps most concerning is the development of secondary degenerate specificities as a result of the affinity enhancement. Depending on the mechanism, targeting of self-tissues can be defined as on-target or off-target. On-target recognition of normal tissues can be operationally defined as the recognition and destruction of normal tissues that either express lower (compared to tumor) levels of the target antigen or express a different antigen that contains the identical epitope as that recognized by the parental T cells (TCR-based approaches) or by the parental antibody (CAR-based approaches). Off-target recognition of normal tissues can be operationally defined as the recognition and destruction of normal tissues that express a different epitope from that recognized by the original targeting agent. Whereas on-target recognition demands maintenance of the specificity of the original TCR or antibody, off-target recognition covers a spectrum of degeneracy in target epitope recognition, from recognition of variant peptides with a single amino acid difference to non-peptide-dependent allo-recognition of target tissues.

Animal models have played significant roles in terms of providing fundamental insights about engineered T cell potency. However, animal models are fundamentally limiting in terms of addressing systems biology questions about T cell therapies. Beyond the limitation of most animal tumor models not faithfully recapitulating human cancer, for T cell therapy studies in particular, model systems fall short because they fail to recapitulate the integrated biology of an intact human immune system. The issue of off-tumor/on-target and off-tumor/off-target specificity is practically impossible to address in preclinical studies, because animal models are generally noninformative for species-specific toxicity and furthermore toxicity has the potential to be patient specific. Comprehensive repositories of normal human tissue could be used to facilitate the functional screening of the potential for off-target toxicities by genetically engineered T cells. The construction of improved humanized mice or use of large animals such as dogs will probably be instrumental to be able to effectively deal with this ongoing issue (O'Connor et al., 2012).

Increased clinical evaluation of adoptive T cell therapy particularly via engineered lymphocytes has revealed that targeting of normal tissues is more than a theoretic concern for the field, with reported examples of both on- and off-target activity. Because engineering of T cells increasingly incorporates the use of non-physiologically derived receptors, it is entirely possible that the potential for off-target recognition may prove to be the exception rather than the rule. Thus, these early data highlight the real need to develop strategies to mitigate the potential serious adverse events that can result from adoptive transfer of potent T cells; such strategies probably will need to involve more comprehensive screening approaches prior to clinical evaluation of new receptors, as well as potent approaches to effectively ablate T cells should undesired activity be observed. Antibody-based approaches to block target epitope recognition have been explored (Lamers et al., 2013).

As described earlier, adoptive transfer of activated bulk CTL has been associated with development of an autologous GVHD syndrome with rash and colitis in about 25% of treated patients (Rapoport et al., 2009). This event probably represents

the effect of activated T cells being sensitized and subsequently triggered to recognize self-antigen-derived peptides in the setting of depleted Treg cells.

On-target toxicity has been reported in cases with T cells engineered with a TCR specific for the carcinoembryonic antigen resulting in severe inflammatory colitis resulting from expression of target antigen in normal colon (Parkhurst et al., 2011). Similarly, TCRs targeting melanoma differentiation antigens destroyed normal melanocytes in the skin, ears, and eyes (Johnson et al., 2009) and more recently with CAR T cells targeting carboxy-anhydrase-IX (CAIX), where on-target destruction of biliary duct epithelial tissue was documented (Lamers et al., 2013).

Severe off-target off-tumor toxicities after infusion of T cells expressing non-physiologically generated TCR have been recently reported (Morgan et al., 2013). Neurologic toxicity in four cases including two deaths were observed in a phase I trial with a MAGE-A3-specific receptor initially generated in HLA-A*02 transgenic mice, with toxicity resulting from the unexpected expression of epitopes derived from other members of the MAGE cancer testis family in the CNS. Notably, clinical regressions of disease were also reported in this study. Severe cardiac toxicity was observed in two cases after administration of T cells expressing an affinity-enhanced TCR that was derived from an HLA-A*01-restricted TCR that was originally specific for MAGE A3. Off-tumor and off-target recognition of titin was demonstrated in these cases (Linette et al., 2013). The engineered TCR was shown to react with MAGE A3 and titin, whereas the parental TCR reacted only with MAGE A3.

An additional to-date-theoretical concern is that engineered T cells may pair with endogenous TCR chains, generating novel specificities (Bendle et al., 2010). This has not yet been observed in clinical trials.

No Pain, No Gain: The Biological Consequences of Potent Targeting of Large Tumor Masses

There are a number of differences between the responses elicited by therapeutic tumor vaccines and adoptive T cell transfer therapy. The response to tumor vaccines and checkpoint blockade often requires several months to become apparent; in contrast, the response to T cell transfer is most often observed in days to weeks. One predicted consequence of triggering rapid and potent antitumor immunity is the development of a generalized proinflammatory immune state. The proinflammatory state can be a consequence of target antigen-driven activation of infused T cells or a result of secondary immune activation triggered by the primary T cell activation event. A series of recent clinical reports have described adverse events associated with adoptive T cell transfer and attributed this to the induction of a proinflammatory immune state. These events are temporally overlapping and may in fact influence each other; nonetheless, they can be distinguished on the basis of hallmark features.

Cytokine release syndrome (CRS) refers to the production of proinflammatory cytokines as a direct consequence of T cell triggering. Hallmark features of the syndrome include a systemic cytokine profile similar to that in response to acute infection, hypotension, and high-grade cyclical fevers. Recent reports have documented CRS in adoptive T cell therapy trials as a consequence of on-target T cell activation (Brentjens et al., 2013; Kalos et al., 2011; Kochenderfer et al., 2012). The cytokine patterns are

similar in adults and children (Grupp et al., 2013; Kalos et al., 2011) and occur several days to weeks after infusion of the T cells. Notably, with one exception (Morgan et al., 2010), cytokine storm, i.e., the immediate release of cytokines after infusion of T cells, has not occurred.

Tumor lysis syndrome (TLS) refers to a combination of metabolic complications that occurs as a result of the destruction of large amounts of tumor and the systemic release of potassium, phosphate, and nucleic acid, which leads to acute renal failure. Hallmark clinical features include hypotension, cardiac arrhythmias, and changes in serum biochemistry including elevations in creatinine, uric acid, potassium, and phosphorus. Delayed TLS has been reported and observed as a result of adoptive T cell transfer of CAR T cells engineered to target CD19-positive malignancies; in this case, TLS occurred 3 weeks after infusion of gene-modified T cells and was coincident with the peak of in vivo T cell expansion and tumor elimination (Porter et al., 2011). In a subsequent case, TLS has been observed as late as 51 days after infusion of CAR T cells.

Macrophage activation syndrome (MAS) and the closely related syndrome hemophagocytic lymphohistocytosis (HLH) is a severe and potentially life-threatening condition typically associated with systemic onset juvenile autoimmune disorders (Tang et al., 2008). Hallmark clinical features of the syndrome include high fevers, pancytopenia, hemophagocytosis in bone marrow accompanied by dramatically elevated ferritin and C-reactive protein levels, activation and proliferation of macrophages and T lymphocytes, and systemic elevations in IFN- γ , GM-CSF, soluble CD163, soluble IL-2 receptor, and IL-6. MAS/HLH has been reported to be associated with administration of blinatumimab, a bispecific T cell engaging antibody (BiTe) that activates T cells to CD19-positive targets in vivo, as well as with the adoptive transfer and potent antitumor activity of CAR targeting pre-B cell acute lymphocytic leukemia (Grupp et al., 2013). In both of these cases, MAS was reversed by the administration of the anti-IL6 receptor antibody tocilizumab. The mechanism of this syndrome remains to be defined. A similar disorder occurs in mice after repeated stimulation through TLR9 (Behrens et al., 2011).

Controversies and Future Directions in the Field

Clinical data generated principally over the past 5 years suggest that we are at the threshold of a golden era for adoptive T cell therapy, where advances in basic immunology have informed the development of a new field of synthetic immunology that may increase the potency of approaches that target cancer. Despite the early successes, a number of fundamental questions still remain to be resolved before widespread implementation of this approach to treat cancer.

With regard to the infused cell product, issues related to the quality, quantity, nature of ex vivo manipulation, and mechanism of genetic modification are essential to address. With regard to the nature of the infused product, questions about whether the most effective strategies will utilize bulk expanded cells or more defined subpopulations of cells such as central and/or effector memory subsets (Turtle and Riddell, 2011), virus-specific T cells (Pule et al., 2008), or potentially products derived from engineered T cell stem cell precursors (Gattinoni et al., 2011) will be important to resolve. Data from recent trials demonstrate that potent and persistent antitumor activity can be gener-

ated through the infusion of small numbers of engineered T cells (Grupp et al., 2013; Kalos et al., 2011), suggesting that quality rather than quantity of infused product may be an attribute critical for potent antitumor activity.

With regard to the tumor, essential questions related to the relevance of tumor burden remain to be addressed. Adoptively transferred T cells make complex “decisions,” sensing multiple inputs and responding to tumor with multiple effector functions including proliferation and functional differentiation. As a result, there is the theoretical consideration that the most effective T cell activation may require higher tumor burden and that therapies may paradoxically be less effective or require higher doses at earlier stages of disease. Corollary issues related to the impact of tumor-driven immunosuppressive mechanisms, involving both surface receptors and soluble mediators, will be important to unravel.

A fundamental and perhaps controversial issue to address in the field is the need for long-term persisting memory T cells in patients. The crux of this issue relates to balancing the need for defining the nature of sterilizing cures in the context of persisting memory T cells and tumor dormancy. Relevant to this issue is that human tumors can remain dormant for at least 16 years (MacKie et al., 2003). In our ongoing clinical studies with CAR engineered cells that target CD19, patients remain disease free and in molecular remission with persisting engineered T cells for at least 2 years after treatment, but also with ongoing B cell aplasia resulting from targeting of normal CD19-positive B cells, highlighting the practical necessity to eventually ablate engineered cells and enable normal B cell reconstitution.

An opportunity for adoptive T cell therapy will be strategies to combine with other antitumor therapies. In particular, therapeutic vaccination, checkpoint inhibition, agonistic antibodies, small molecule inhibitors of tumors, and targeting of tumor stroma and neo-vasculature may augment current adoptive transfer technology.

Finally, engineered T cells are poised as a disruptive technology advance. The complexity of cells and the challenge of controlling T cells after the introduction of synthetically derived receptors in a therapeutic setting raises new and daunting scientific, regulatory, economic, and cultural obstacles to the establishment of engineered T cells as a widespread and viable pharmaceutical platform. It is encouraging that the US Food and Drug Administration has been proactive in balancing the benefits and risks of subjects in cell-therapy clinical trials (Au et al., 2012).

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